Plant Cell Reports (2000) 19:333–338

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Inhibition of fungal growth in planta and in vitro by transgenic tobacco expressing a bacterial nonheme chloroperoxidase gene

Received: 10 March 1999 / Revision received: 22 June 1999 · Accepted: 5 July 1999

Abstract Transgenic tobacco plants producing chloroperoxidase (CPO-P), encoded by a novel gene from *Pseudomonas pyrrocinia*, were obtained by *Agrobacterium*-mediated transformation. Successful transformation was shown by PCR, Southern, northern and western blot analyses, and assays of CPO-P enzyme activity. Extracts from plants transformed with the CPO-P gene significantly reduced *Aspergillus flavus* colonies by up to 100% compared with extracts from control plants transformed with pBI121. Compared with controls, the transformed plants showed increased disease resistance in planta against a fungal pathogen, *Colletotrichum destructivum,* the causal agent of tobacco anthracnose.

Key words Chloroperoxidase · Disease resistance · Haloperoxidase · Reactive oxygen species · Tobacco

Abbreviations *CaMV*: Cauliflower mosaic virus · *CPO*: Chloroperoxidase · *CPO-P*: Chloroperoxidase from *Pseudomonas pyrrocinia* · *HPO*: Haloperoxidase · *MCD*: Monochlorodimedon · *MPO*: Myeloperoxidase · *ROS*: Reactive oxygen species

Introduction

Living organisms possess several inherent defense mechanisms to fight microbial pathogens. One of the chronologically earliest responses to invasion is a respiratory burst that produces two reactive oxygen species (ROS) that are microbicidal: superoxide and hydrogen peroxide (Mehdy 1994; Wojtaszek 1997). Both plants and animals generate ROS in response to microbial invasion, but only animals contain myeloperoxidase (MPO) which converts hydrogen peroxide to a much stronger antimicrobial compound, hypochlorous acid (HOCl; Klebanoff 1980): $H_2O_2 + HCl \rightarrow H_2O + HOCl$. Since plants do not have MPO, transformation with MPO might confer increased disease resistance to phytopathogens (Jacks et al. 1991). However, even with successful transformation, MPO and most other haloperoxidases (HPO) require species-specific heme-containing prosthetic groups to catalyze the redox reaction (Pfeifer et al. 1992; Pée 1996). These heme derivatives are not available in plants. Thus, a transformation scheme involving MPO or HPO would require the addition of a plethora of genes coding for the multicomponent synthesis of each specific heme group. An alternative would be transformation with a gene encoding a functional yet heme-free enzyme. Picard et al. (1997) found that certain bacterial HPOs do not require heme prosthetic groups or even metal ion cofactors. Furthermore, these HPOs catalyze not only the formation of hypohalites from hydrogen peroxide but also the formation of peracetic acid. Both hypohalites and peracetic acid are strong antimicrobial agents (Pée 1996).

In this study, we introduced a gene for metal-free, nonheme bacterial chloroperoxidase (CPO, EC 1.11.1.10) from *Pseudomonas pyrrocinia* (CPO-P) into tobacco plants by *Agrobacterium*-mediated transformation and examined its effect on the growth of certain phytopathogens.

Communicated by I.K. Vasil

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Materials and methods

Cloning and manipulations

Total genomic DNA was isolated from tobacco leaf tissue using the method of Paterson et al. (1993). Total RNA was isolated

from leaf material according to the method of Logemann et al. (1987). The *cpo* gene from *P. pyrrocinia* (Wolframm et al. 1993) was subcloned into the plant binary vector KYLX 7.1 which places expression of the gene under the control of the CaMV 35S promoter (Schardl et al. 1987). Synthetic oligonucleotide primers were designed for amplification of the *cpo* ORF based on N-terminal and C-terminal sequence data. The primers had *Hin*dIII (CPO-N) and *Sac*I (CPO-C) restriction enzyme sites engineered into them to facilitate subcloning into the binary vector. Using plasmid pHW321 (Wolframm et al. 1993) harboring the *cpo* gene as template, the primers CPO-N, 5′-AAGCTTTGCCAT ACGT-CACTACG-3′ and CPO-C, 5′-GAGCTCTACGCCTGCACG-AACG-3′ were used to amplify the *cpo* coding region using *Pfu* polymerase (Stratagene, La Jolla, Calif.). The 836 bp PCR product was subcloned into vector pCRScript-Cm (Stratagene) and sequenced to confirm fidelity of the PCR reaction. The *cpo* coding region was released from pCRScript-CPO DNA by *Hin*dIII-*Sac*I digestion and subcloned into *Hin*dIII-*Sac*I digested KYLX 7.1 vector DNA (Schardl et al. 1987). The resulting vector, KYLX-*cpo*, was transformed into electrocompetent *Agrobacterium tumefaciens* LBA 4404 cells (Gibco-BRL, Bethesda, Md.) via electroporation using a Bio-Rad Cell-Porator (Bio-Rad, Hercules, Calif.) according to the manufacturer's procedure.

Plant transformation

Transformation of tobacco (*Nicotiana tabacum* cvs. Xanthi and SR-1) was accomplished using the *A. tumefaciens*-mediated leaf disk transformation system (Horsch et al. 1985). The selection medium (Murashige and Skoog 1962; MS) supplemented with 6-benzylaminopurine (0.75 mg l^{-1}) also included kanamycin (200 mg l^{-1}) for selection of transformed cells. The kanamycin-tolerant shoot buds were transferred to MS medium containing kanamycin (50 mg l^{-1}) and the rooted plants were subsequently transferred to soil for further evaluation in a growth chamber (28 °C, 16 h photoperiod per day). The potted plants were also assayed for the presence of NPT II protein by ELISA (Rajasekaran et al. 1996). Plants regenerated from a parallel transformation experiment with pBI121 served as negative controls in the molecular and antifungal analyses.

PCR and Southern blot analysis of plant genomic DNA

To determine if the CaMV 35S-*cpo* T-DNA region had successfully integrated into the plant genome, a 640 bp region spanning from within the CaMV 35S promoter to 400 bp into the *cpo* gene was PCR amplified from total plant genomic DNA. The CaMV 35S primer, 5′-TCATTGCGATAAAGGAAAGGCC-3′ and the CPO internal primer, 5′-GATTCGGTTTTCAGCATCAGC-3′ were used to amplify plant genomic DNA using AmpliTaq polymerase (Stratagene). Thermocycler (MJ Research, Watertown, Mass.) parameters were as follows: 1 cycle of 95 °C, 5 min; 58 °C, 1 min; 72 °C, 30 s; 1 cycle of 95 °C, 1 min; 60 °C, 1 min; 72 °C, 30 s; 32 cycles of 95 °C, 1 min; 65 °C, 1 min; 72 °C, 30 s. This was followed by a final extension of 72 °C for 1 min. PCR products were analyzed by electrophoresis on a 1% agarose gel followed by ethidium bromide staining.

For Southern blot analysis, tobacco genomic DNA (20 μg) was digested to completion with *Eco*RI and electrophoresed on a 1% agarose gel. DNA was transferred to nylon membranes (Schleicher & Schuell, Keene, N.H.) by vacuum transfer and hybridized with the 836 bp random-primed, ^{32}P-labelled, *cpo* gene PCR product.

Northern blot analysis of plant RNA

Total plant RNA was isolated and electrophoresed on a 2.2 M formaldehyde/agarose gel according to standard methods (Ausubel et al. 1993). RNA was transferred to a nylon membrane by vacuum transfer and hybridized with the 836 bp ^{32}P-labelled, *cpo* gene PCR product.

Western blot analysis of *cpo* expression

Plant leaf tissue was ground to a fine powder with liquid nitrogen in the presence of an extraction buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 5% 2-mercaptoethanol). Cell debris was removed by centrifugation at 13,000 *g* at room temperature for 5 min. Supernatant was collected and the total protein in each sample was determined using the Bio-Rad Protein Assay kit (Bio-Rad) with bovine serum albumin as a standard. Bromophenol blue (0.05%) was added to the protein samples which were then separated on a 10% polyacrylamide gel along with protein molecular weight standards (14.4–200 kDa Rainbow Markers, Amersham International, Amersham, UK). Following electrophoresis, the proteins were transferred to PVDF membranes (ICN, Costa Mesa, Calif.) using a Semi-Phor electroblotter (Hoefer Scientific, San Francisco, Calif.). CPO-P protein was detected using Bio-Rad immunodetection procedures with CPO-P polyclonal antiserum (a gift from K.-H. van Pée, 1:200 dilution) and goat-anti-rabbit, alkaline phosphatase secondary antibody conjugate (Bio-Rad, 1:3000 dilution). Nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate reagents were used for color development.

Enzyme assay for CPO-P in leaf extracts

Young expanding leaves were pulverized in liquid N_2 with two parts (v/w) of 1.0 M Na acetate buffer (pH 5.5) and centrifuged at 15,000 *g* for 10 min. Then 0.1 part (w/v) of insoluble, high MW, cross-linked polyvinylpyrrolidone was added to the liquid supernatant and the mixture was centrifuged again at 15,000 *g* for 10 min. The supernatant was assayed for halogenating and peroxidative activities.

Halogenating activity of CPO-P in leaf extracts was assayed with 0.8 M Na acetate buffer, pH 5.5, containing 44 μM monochlorodimedon (MCD), 7.2 mM H_2O_2, 82 mM NaBr and 8.9 mM NaN_3 (Picard et al. 1997). The reaction was initiated by the addition of leaf extract and the decrease in absorbance at 290 nm due to halogenation of MCD was monitored.

Peroxidase activity in leaf extracts was assayed at 470 nm with 0.8 M Na acetate buffer, pH 5.5, containing 7.0 mM *o*-methoxyphenol (guaiacol) and 7.2 mM H_2O_2 (George 1953).

In vitro analysis of antifungal activity of plant extracts to *Aspergillus flavus*

The inhibitory activity of extracts from tobacco plants transformed with the *cpo* gene was assessed in vitro following the method of DeLucca et al. (1997).

Plant homogenates were prepared by grinding tobacco leaves with liquid N_2 with no buffer added. Ground tissues were then centrifuged at 8200 *g* for 10 min at room temperature and extract collected from each sample. Control samples consisted of extract from tobacco plants transformed with pBI121. Pre-germinated conidial suspensions (10^5 conidia/ml) of *A. flavus* (25 μl) were then added to 225 μl of plant extract, mixed, and incubated for 60 min at 30 °C. Three 50 μl aliquots from each sample tested were then spread onto PDA plates and incubated at 30 °C for 24 h and fungal colonies enumerated. Antifungal assays were conducted at least three times. One-way ANOVA was used to determine the effect of extracts collected from transformed plants on germinating conidia. Mean separations were performed using the method of Tukey (Sokal and Rohlf 1981).

In planta antifungal assay for tobacco anthracnose resistance

Colletotrichum destructivum (ATCC 42492) inoculum was prepared by flooding a 7-day-old culture of the fungus with 9 ml of sterile distilled water and gently removing spores with a sterile pipette tip to yield a final inoculum density of approximately

1×10^6 spores/ml. R_0 transgenic tobacco plants, grown in an environmentally controlled growth chamber, were inoculated by placing 10 drops of 10 μl each onto the adaxial surface of young, but fully developed tobacco leaves. In each experiment, leaves of at least three pBI121-transformed tobacco plants were also inoculated to serve as a negative control. Inoculated leaves were then covered with a plastic bag for 4 days. Disease severity was scored for each inoculation site of each leaf separately 7 days after inoculation using an arbitrary scale from 0 (no visible symptoms) to 5 (necrotic lesion >5 mm in diameter). One-way ANOVA was used to determine the effect of transgenic plants on disease severity. Mean separation was performed using the method of Tukey (Sokal and Rohlf 1981).

Results

Transformation of tobacco

Each leaf disk produced at least five transformed shoots in the presence of toxic levels of kanamycin (200 mg l^{-1}), which developed roots in growth media containing inhibitory levels of kanamycin (50 mg l^{-1}). The putative antibiotic-resistant plantlets were assayed for the presence of NPT II. To obtain different individual transformants only one NPT II-positive plantlet from each leaf disk was transferred to the greenhouse. All of the ten transgenic tobacco plants of cv. Xanthi carrying the *cpo* gene were morphologically similar to non-transformed controls with respect to flowering and seed set. Six R_0 plants, labeled H1, H2, H3, H5, H6 and H7, were utilized in the disease resistance assays. Using a different variety (SR-1), the transformation and the disease resistance assays were duplicated with a second set of ten R_0 transformants with similar results (data not shown).

PCR and Southern blot analysis of plant genomic DNA

Agarose gel electrophoresis of PCR products from the transgenic plant samples showed the expected PCR product of 640 bp representing the region of DNA spanning from within the CaMV promoter to 400 bp into the *cpo* gene (data not shown). The no-DNA sample and negative control DNA from pBI121 transformed sample did not show this product. Southern transfer of the PCR samples and hybridization with the radiolabeled *cpo* gene probe demonstrated a band of hybridization at about 640 bp, confirming that the PCR product did represent the CaMV 35S-*cpo* gene region in all the putative transgenic plants (not shown). No signal was detected in the no-DNA or negative tobacco DNA control lanes. Southern blot analysis of *Eco*RI-digested plant genomic DNA from H1, H2, H5 and H7 showed that all the transgenic plant tissues gave a single hybridization signal with the radiolabeled *cpo* gene probe (Fig. 1). The pBI121-transformed control lane did not show a hybridization signal with the probe, as expected. The length of the fragment from the T-DNA insertion site to the closest *Eco*R1 of the surrounding plant DNA was approximately the same for H1 and H5 (Fig. 1).

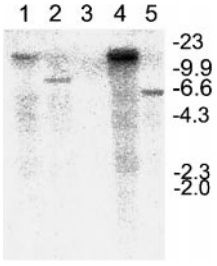

Fig. 1 Southern hybridization of tobacco genomic DNA with a radiolabeled cpo gene probe. Tobacco genomic DNA (20 μg) was digested to completion with *Eco*RI, electrophoresed, blotted to nylon membrane and hybridized with ^{32}P-labelled 836 bp *cpo* gene PCR probe. *Lanes*: *1* H1, *2* H2, *3* negative control plant transformed with pBI121, *4* H5, *5* H7. *Hin*dIII-digested lambda DNA (in kbp) was used as molecular size standard

With the possible exception of plant H5, all of the other transformed plants appeared to have only one copy of the cpo gene integrated into the plant genome. The intensity of the signal in plant H5 DNA is possibly due to loading of more DNA, as confirmed by Ethidium bromide staining prior to Southern transfer.

Northern blot analysis of *cpo* transcripts

Hybridization of total RNA with the radiolabeled *cpo* gene ORF fragment demonstrated the presence of *cpo* transcripts in all samples tested except the negative control (Fig. 2). Highest transcript levels were observed with sample H7 followed by sample H1. Very little *cpo* transcript was detected in samples H2 and H5 and this was apparently due to degradation of the *cpo* mRNA, as a significant amount of smearing of the signal was observed.

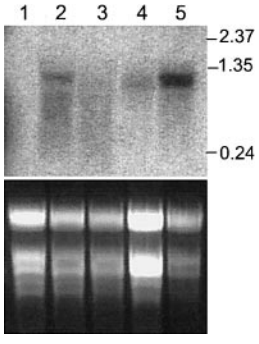

Fig. 2 Northern hybridization analysis of transcripts probed with radiolabeled cpo gene. Total RNA (10 μg) was separated electrophoretically, blotted, and hybridized with ^{32}P-labelled 836 bp *cpo* gene PCR product. *Lanes*: *1* tobacco negative control plant transformed with pBI121, *2* H1, *3* H2, *4* H5, *5* H7. Ethidium bromide stained RNA is shown in the *bottom panel* to indicate the amount loaded. The position of molecular size markers (in kb) is shown

Western blot analysis of CPO-P protein

All transgenic plant samples demonstrated a band of reactivity at approximately 31 kDa (Fig. 3). This band aligned exactly with the protein sample from the negative control plant transformed with pBI121 that had 10 ng of purified *cpo* protein added to it.

Evaluation of CPO-P activity in transgenic leaf extracts

Halogenating activity of CPO-P was observed in transgenic tobacco plants but not in the negative control plants (Table 1). Plants with increased enzymatic activity tended to have correspondingly increased antifungal activity (see below).

The validity of the assay procedure for CPO-P activity in transgenic tobacco was established in the following manner. Complete recovery of enzymic activity was obtained in leaf extracts from negative control plants spiked with authentic CPO-P, which is consistent with physiological compatibility of the plant cellular milieu for CPO-P. Furthermore, NaN_3 completely inhibited endogenous peroxidase activity in leaf extracts. These results show that artifacts from endogenous peroxidases were absent and that only non–heme CPO-P activity was being measured.

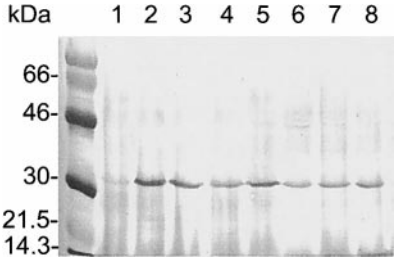

Fig. 3 Western blot analysis of CPO-P protein from tobacco tissue. Crude protein extracts of tobacco leaf tissue were separated on a 10% SDS-polyacrylamide gel and electroblotted to PVDF membrane. The membrane was treated with a 1/200 dilution of CPO-P polyclonal antiserum. *Lanes*: *1* negative control plant transformed with pBI121, 2 H1, *3* H2, *4* H3, *5* negative control plant sample with 10 ng pure CPO-P protein added, *6* H5, *7* H6, *8* H7. Molecular weight standards (in kDa) are shown

Table 1 CPO-P activity in transformed and non-transformed tobacco (cv. Xanthi). Halogenating activity in leaf extracts was measured as a decrease in absorbance at 290 nm due to halogenation of MCD

Leaf source		CPO-P activity (Δ Absorbance min^{-1} mg^{-1})[a]
Control plants transformed with pBI121		0
Transformed with CPO-P	H1	0.52
	H2	1.26
	H3	0.28
	H5	0.21
	H6	0.65
	H7	1.15

[a] Values are averages of three determinations; ranges were within 5% of the averages

In vitro antifungal activity of transgenic tobacco extracts

Plant extracts from tobacco plants transformed with the *cpo* gene significantly reduced ($P<0.05$) the number of fungal colonies arising from germinating conidia of *A. flavus* compared with the extracts from the pBI121-transformed control (Fig. 4). Extracts from the transformed plants reduced the number of *A. flavus* colonies by up to 100% compared with the negative tobacco control (Fig. 4).

In planta anthracnose resistance

Leaves inoculated with *C. destructivum* developed anthracnose lesions within 48–72 h after inoculation. One-way analysis of variance indicated that all the transformants showed significantly reduced ($P<0.05$) anthracnose severity compared with the controls (Figs. 5, 6). Lesions developed more slowly on plants transformed with *cpo* than on the non-transformed or pBI121-transformed controls (data not shown).

Discussion

Compared with most other HPOs, CPO-P does not contain a heme, making it very amenable for transfor-

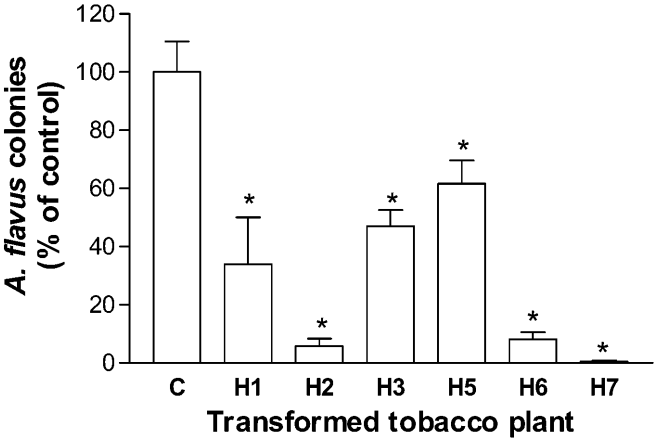

Fig. 4 Inhibition of germinated spores of *Aspergillus flavus* by leaf extracts from tobacco plants transformed with the *cpo* gene construct compared with the negative control (*C*). * denotes significant reduction ($P<0.05$) in the number of *A. flavus* colonies compared with control plants transformed with pBI121. Mean values of at least two separate analyses, with a minimum of three replicates in each, are given. *Error bars* indicate standard error of means. Mean separation was performed by Tukey's method

Fig. 5 Tobacco leaves (cv. Xanthi) from a control tobacco plant transformed with pBI121 (left) and a transgenic plant (H7; right) showing anthracnose symptoms 7 days after inoculation

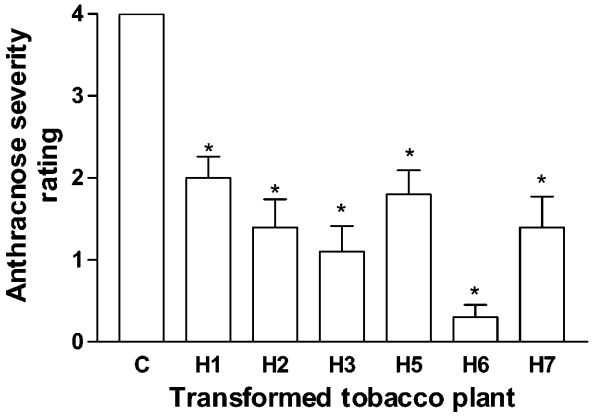

Fig. 6 Anthracnose severity among tobacco plants transformed with the *cpo* gene construct compared with the negative control (*C*). * indicates significant difference ($P<0.05$) in anthracnose severity from the negative control plant as determined by Tukey's ANOVA. Mean values from ten inoculation sites are given

mation into plant systems. Ours is the first report on the utility of a HPO gene for pathogen defense in plants. We have demonstrated that expression of a gene encoding CPO-P results in the significant reduction in anthracnose severity caused by the tobacco pathogen *C. destructivum* (Figs. 5 and 6). We have also demonstrated that plants expressing a gene encoding CPO-P strongly inhibits the in vitro growth of the mycotoxin producing fungus *A. flavus* (Fig. 4). The underlying mechanism of inhibition of growth of phytopathogens by transgenic plants producing CPO-P is not well understood. Reactive oxygen species such as H_2O_2 possess antimicrobial properties (Mehdy et al. 1996; Wojtaszek 1997) and are involved in eliciting a hypersensitive response in numerous plant-pathogen systems (Lamb and Dixon 1997; Low and Merida 1996; Mehdy 1994; Mehdy et al. 1996; Tenhaken et al. 1995). Recently, Jacks and Davidonis (1996) demonstrated that cultured cotton cells elicited with fungal cell wall components have the potential to generate 1 M H_2O_2 within 15 min. However, initial protection from the pathogens by the ROS is apparently not sufficient to prevent infection. Attempts to increase the level of hydrogen peroxide has proven to be useful in increasing resistance to plant diseases. For example, Wu et al. (1995) showed that expression of an *Aspergillus niger* gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants enhanced disease resistance to phytopathogens. Thus, the importance of ROS, including H_2O_2, in the plant defense response to pathogenic infection is now well established (Wojtaszek 1997). HPOs carry this defense mechanism a giant step further by utilizing the naturally occurring defense product, H_2O_2, to generate far more lethal microbicidal compounds. HPOs catalyze the peroxidation of halides to form hypohalites: $H_2O_2 + X^- \rightarrow H_2O + {}^-OX$, where X^- is a halide other than fluoride, and ^-OX is a hypohalite (Pée 1996). This reaction, catalyzed by MPO, is used by animal cells to kill invading microbes (Klebanoff 1980) and is lethal to *A. flavus* (Jacks et al. 1991). The particular haloperoxidase we employed, CPO-P (Wiesner et al. 1988; Wolframm et al. 1993), also catalyzes a newly discovered biological reaction – the formation of peracetic acid (Picard et al. 1997; Pée 1996): $AcOH + H_2O_2 \rightarrow AcOOH + H_2O$, where AcOH is acetic acid and AcOOH is peracetic acid. How much each of these reactions contributes to antimicrobial activity in transgenic plants is currently unknown. In addition, CPO-P exhibits esterase activity (Pée 1996) which might be responsible for some of the observed antimicrobial activity.

The utility of the CPO-P gene in disease control has broader implications. For example, leaf extracts from the transgenic tobacco plants are lethal to germinated spores of *A. flavus* (Fig. 4), the fungus that produces aflatoxin during growth on crops such as cotton, peanut and corn. In summary, the control of aflatoxigenic *A. flavus* by CPO-P makes it an attractive candidate for genetic engineering in several important cultivated crops to enhance food and feed safety.

Acknowledgements The authors thank K.-H. van Pée for the *cpo* gene and the cpo-P polyclonal antiserum, Tony DeLucca for advice on antifungal assays, Caryl Chlan for Xanthi tobacco plants, and Pamela Harris, Neel Barnaby and Alicia Waits for technical assistance.

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